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1978 RESEARCH PROGRESS REPORT

NASA GRANT 7300

**The Role of Cell Membranes in the Regulation of
Lignification in Pine Cells**

(NASA-CR-157156) THE ROLE OF CELL MEMBRANES
IN THE REGULATION OF LIGNIFICATION IN PINE
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1977 Research Progress Report

- I. Research Title: The Role of Cell Membranes in the Regulation of Lignification in Pine Cells
- II. Principal Investigator: Donald L. Hendrix, Department of Biology, the University of Houston, Houston, Texas, 77004
713/749-3792
- III. Objectives and Research Effort:

Both lignification and various geopotential responses in plants share an involvement with plant cell membranes. This research is designed to test the hypothesis that cellular membranes are involved in the control of lignification and therefore involved in the gravity response known as reaction wood formation. Previous work showed that lignin precursors and enzymes in the lignin pathway are compartmentalized by cellular membranes; the proposed research will evaluate the contribution of this compartmentation to lignification and the compression wood response in Pinus elliotii. Emphasis will be placed upon the enzyme phenylalanine ammonia-lyase (PAL) because of its suspected role as a control point in lignin synthesis.

Specific objectives of this project include the confirmation of the identity of pine cell membranes bearing PAL activity, the isolation of a plasma membrane preparation from pine cells for the testing of this organelle as a regulatory barrier in lignification, the measurement of the geopotential effect in pine stem and the incorporation of these results into a model to describe and predict the interaction of gravity and lignification of higher plants. The past few months our laboratory has concentrated upon the identification of membrane fractions bearing PAL activity and determining base line electrical potential data (i.e., transcellular membrane potentials) against which to compare geostimulated plant values to detect the subtle geopotential effect in Pinus elliotii stem.

IV. Methodology Used/to be Used to Achieve Objectives:

Pine cells, obtained primarily from seedlings grown upon a combination of vermiculite and absorbent paper, are being fractionated after disruption by differential and isopycnic centrifugation on sucrose gradients. The type of disruption technique has been found to be critical; we usually employ a Brinkmann Polytron for purposes of cell disruption. We have concentrated upon differential centrifugation at 270, 13,000, 40,000 and 80,000 times gravity (g). These centrifugation regimes result in four pellets, a high speed supernatant and a crude homogenate fraction. Each fraction is being characterized for total and specific PAL activity, as well as the susceptibility of each of these activities to metabolites from various metabolic paths following PAL to determine the possible relationship of the PAL in these fractions to these paths.

We are attempting to prepare a relatively enriched fraction of plasma membrane from Pinus by localization of marker enzymes on continuous sucrose gradients. Such marker enzymes used to detect this fraction in other higher plant systems include glucan synthetase, a K-stimulated, Mg-activated ATPase and cellulase. Another identifying feature of this membrane is its ability to stain selectively with an electron-dense stain termed the PACP stain, discovered by Roland and Morré. We have shown that this stain also selectively stains the plasma membrane of pine in electron microscopy of intact cells. We will employ the same stain on our enriched fraction as a confirmatory procedure.

In another electron and visible microscopy project, we are tracing the path of lignin precursors into lignin in stems of plants exposed to inclined attitudes with respect to gravity. The technique relies upon autoradiography and was developed for non-gravity stimulated stems by J. D. Pickett-Heaps. Stems will be exposed to radioactive lignin precursor, inclined, and the stem sectioned after embedding in plastic for electron microscopy. These sections will then be used to expose photographic emulsions in order to trace the path of these compounds across the cell in both gravity-stimulated and non-stimulated stems. In this way, we hope to detect differences in lignin synthesis at the organelle/membrane level, under those conditions invoking a reaction wood

response.

In a final project, we are attempting to detect the geo-electrical potential effect in these stems by employing Ling-Gerard microelectrodes in a similar fashion to that used by Etherton and Dedolph with corn coleoptile tissue. Since the electrical potential differences expected are quite small (only a few percent of the total transmembrane potential), we are obtaining a relatively large number of observations of the electrical potential in non-stimulated stem cells, in order to have a reliable baseline against which to compare data from stimulated stems in the next phase of the project. We have found that the potential of pine seedling stem cells can be readily measured by our equipment, using either intact stems, stem segments, or intact seedlings. The electrical measurements for these three tissue types are in fairly close agreement, which is not always true for higher plant tissue. We are presently acquiring the equipment to build a second micromanipulator which will allow the implantation of electrodes into either the top or bottom of a particular cell, which should give us the capability to detect any geo-electrical effect in these plants.

V. Progress Report, Significant Findings, Problems:

In our project to detect PAL in continuous and differential sucrose gradients, we have noted (along with the adjoining laboratory of Dr. J. R. Cowles) that the intact pine cell exhibits PAL activity in the assay techniques used, and must therefore be removed from low speed fractions to prevent artifactual PAL values. If these cells are removed, the 270g fraction shows very little PAL activity (in relation to the total in the crude homogenate); however, the 270g fraction appears to be quite active if it is not filtered. The bulk of the PAL activity appears to be in the high speed supernatant (which we refer to as soluble PAL); however, there is a significant proportion of the total PAL associated with the 270-40,000g pellet (a fraction which we refer to as microsomes). This fraction, containing organelles and organelle membranes, is being characterized by continuous and discontinuous gradient centrifugation. Thus far, we have tentatively identified PAL activity associated with the endoplasmic reticulum, the plasma membrane, the mitochondria and the chloroplast membranes. We are

presently attempting to confirm this localization by utilizing the position of other membrane markers on these gradients.

The assay method used to determine PAL activity in these fractions involves ^3H -phenylalanine. The reaction converts the tritium to tritiated ammonia, and thence tritiated water. This tritiated water is measured after sublimation. Another method of measuring PAL involves the use of ^{14}C -labeled phenylalanine and the extraction of the reaction mixture with toluene. The phenylalanine does not dissolve in toluene, whereas the reaction product, cinnamic acid, is quite toluene soluble. The problem with either assay method, is that other products are formed than just cinnamate, especially in relatively crude membrane preparations. One of these, phenylacetate, is also quite soluble in toluene and has caused the latter assay method to be questioned recently. We have tested both assay methods and find, at least in pine cells, that the two methods give very similar results. Furthermore, that the majority of the radioactive label goes into cinnamate is being established by paper chromatography. The relatively small fraction that ends up in other products is presently being investigated by paper and high pressure liquid chromatography. We would like to know the identity of the products as well as the relative amount of material ending up in other products, so that we can correct our assay methods by this amount.

The high pressure liquid chromatographic separation method, being developed by Dr. Stanley Deming in the Department of Chemistry at the University of Houston, should allow us to answer several important questions. It has been suggested a number of times that the lignification pathway beyond phenylalanine occurs by a multienzyme complex. If such is the case, it seems likely that some of our cellular fractions should be able to produce several products simultaneously! the kinetics of their appearance should be easily detected by sequential sampling with HPLC. Since the metabolites of this path are difficult to separate by other chromatographic methods, HPLC may offer a quick quantitative method of determining the activity of the enzymes in this path found in our cellular fractions.

One problem encountered thus far concerns the availability of machine shop facilities to manufacture needed chambers and for the modification of micromanipulators and microelectrode pulling devices for the electrical potential measurements. The facilities available on campus are understaffed and we cannot locate other facilities which can carry out such mechanical operations. We need to either construct or purchase a Chowdhury-type electrode pulling machine which will be a great aid in the geo-electrical potential measurements; due to the extremely small electrode tips it can produce. Attempts to purchase such a device have, thus far, been unsuccessful.

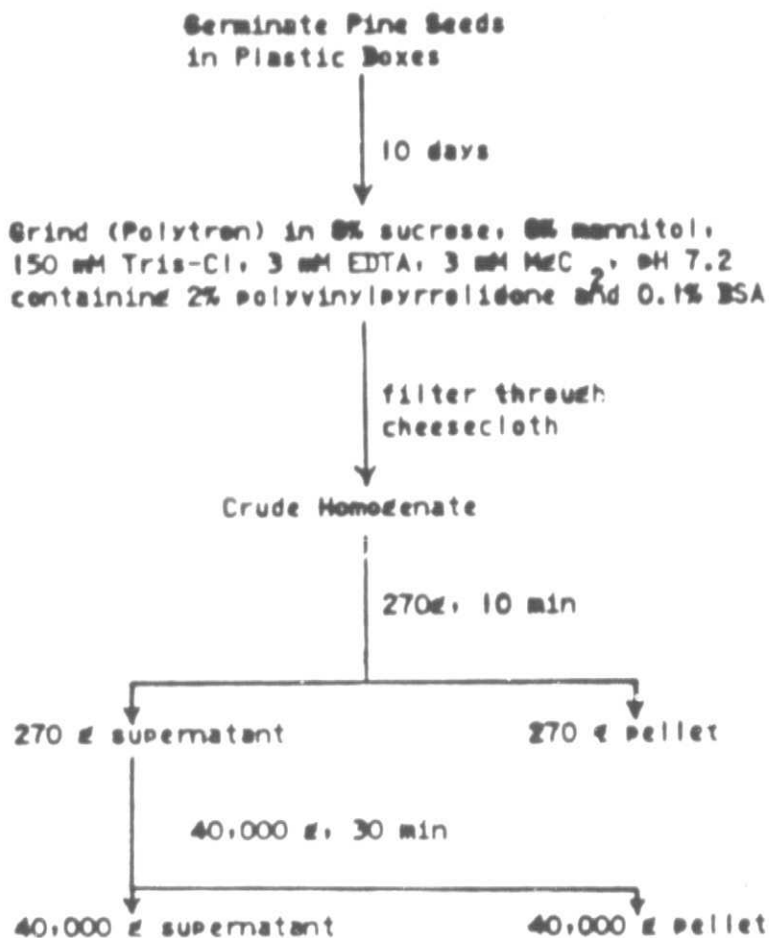
V. Future Plans:

In the next six months- we will:

1. Complete the confirmation of PAL-containing membrane fractions (especially in the microsomal fraction) by electron microscopy and enzyme marker studies.
2. Isolate a plasma membrane preparation from pine stem cells for later in vitro lignin precursor studies.
3. Begin an autoradiographic study of the effect of gravity on the movements of lignin precursors across pine stem cells.
4. Measure the geopotential effect in pine stem cells using Ling-Gerard microelectrodes and a modified electrode manipulator mounted at 45° to the vertical.

APPENDIX

Figure One. Typical flow sheet of Pinus elliotii fractionation.



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FIGURE TWO. Relative PAL activity in cellular fractions prepared essentially as in Fig. One.

PHENYLALANINE AMMONIA LYASE ACTIVITY*			
Fraction	CPM ³ H/ml	CPM ³ H/mg prot	% total PAL
Crude	15,140	793	100 %
270 g	5,400	563	2
13,000 g	24,520	2,595	11
40,000 g	10,750	1,136	3
80,000 g	2,260	508	1
h. s. sup.	10,700	1,174	71

* Assayed by the method of Amrhein, et al., Planta 131: 33-40 (1976)

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OF LOWER QUALITY

Figure Three. Response of the PAL activity associated with various cellular fractions from Pinus elliotii stem cells to various phenolic metabolites. Such inhibitions can help determine the identity of the pathway associated with each PAL fraction.

EFFECT OF METABOLITES ON PAL ACTIVITY IN PINE SEEDLING STEM FRACTIONS

Fraction	Control*	Vanillic Acid	Caffeic Acid	Benzolic Acid	Gallic Acid
Crude	276	332, 120 %**	360, 130 %**	296, 107 %**	245, 89 %**
270 g pel	310	229, 74	193, 50	223, 72	226, 73
40,000 g pel	493	495, 100	342, 69	354, 72	274, 56
40,000 g sup	222	177, 79	270, 122	254, 114	254, 114

* CPM ³H by the assay method of Amrhein, et al., Planta 131: 33-40 (1976).

** % of control activity. Control contained 7% 2-methoxyethanol.

Figure four. Distribution of PAL and other enzymes on a continuous sucrose gradient, at equilibrium. 270-40,000g pellet layered onto the gradient.

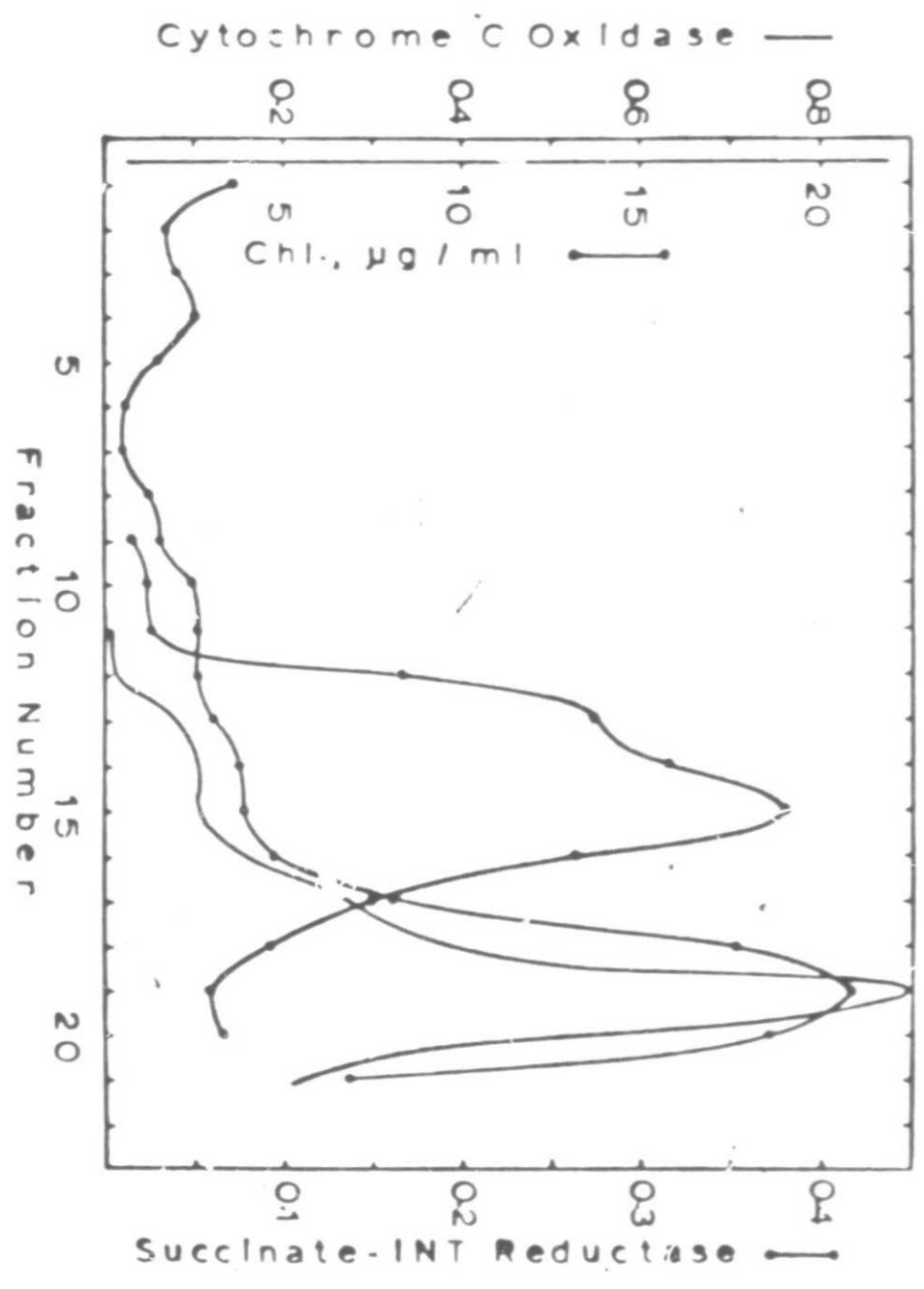
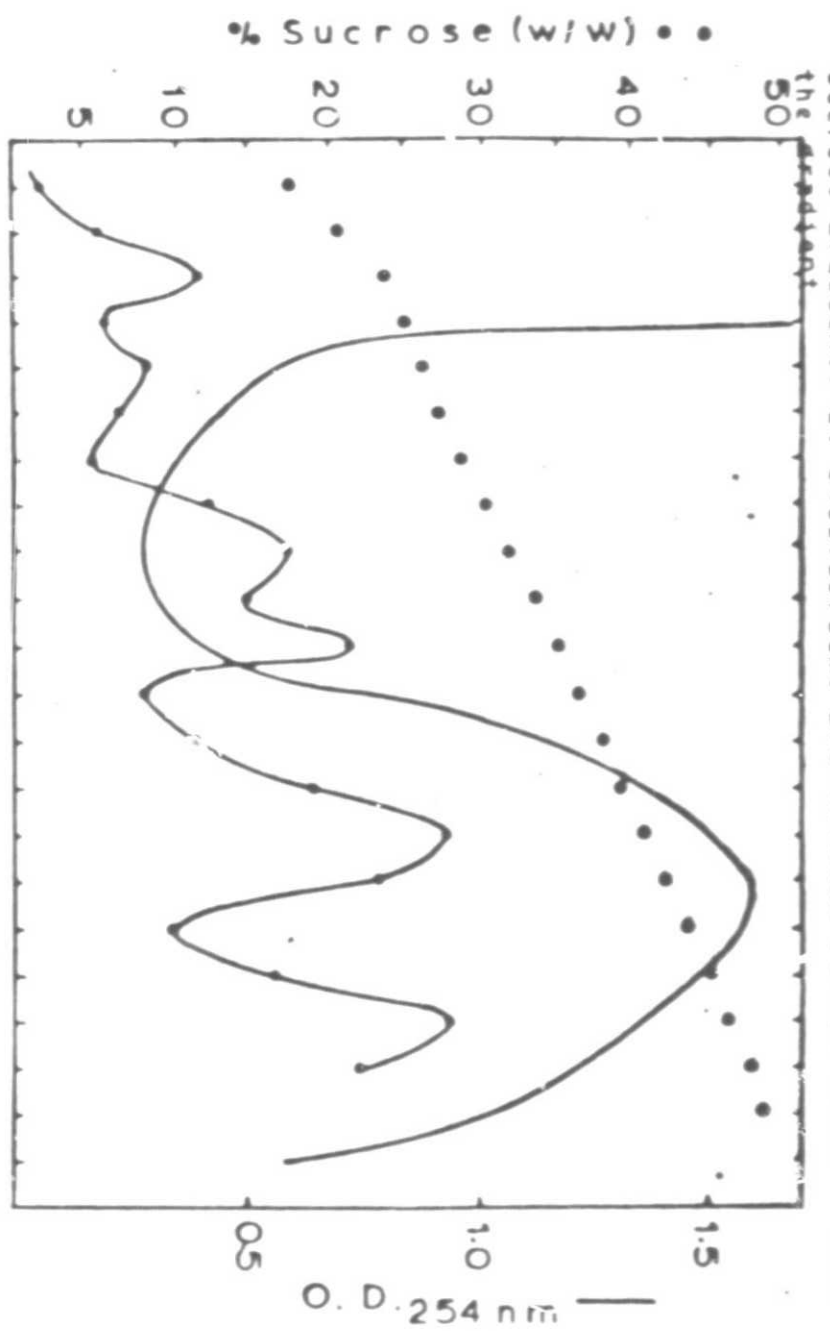


Figure Five. Transmembrane electrical potential of 10 day old *Pinus elliotii* cells, measured in 0.5 mM CaSO_4 and 0.1 mM KCl. The cell exterior is considered to be 0.000 mV.

Seedling Region	Electropotential of Detached Segments	Electropotential of Intact Seedlings
Cotyledons	--	-132 mV
Upper Hypocotyl (Green)	$-111.6 \pm 16.2^* \text{ mV}$	$-107 \pm 1.15 \text{ mV}$
Middle Hypocotyl (Red)	--	$-83 \pm 17 \text{ mV}$
Lower Hypocotyl (Brown)	$-93 \pm 11.9 \text{ mV}$	--
Root (White)	$-76 \pm 12.8 \text{ mV}$	$-80.5 \pm 2.5 \text{ mV}$

* Standard deviation.